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Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences

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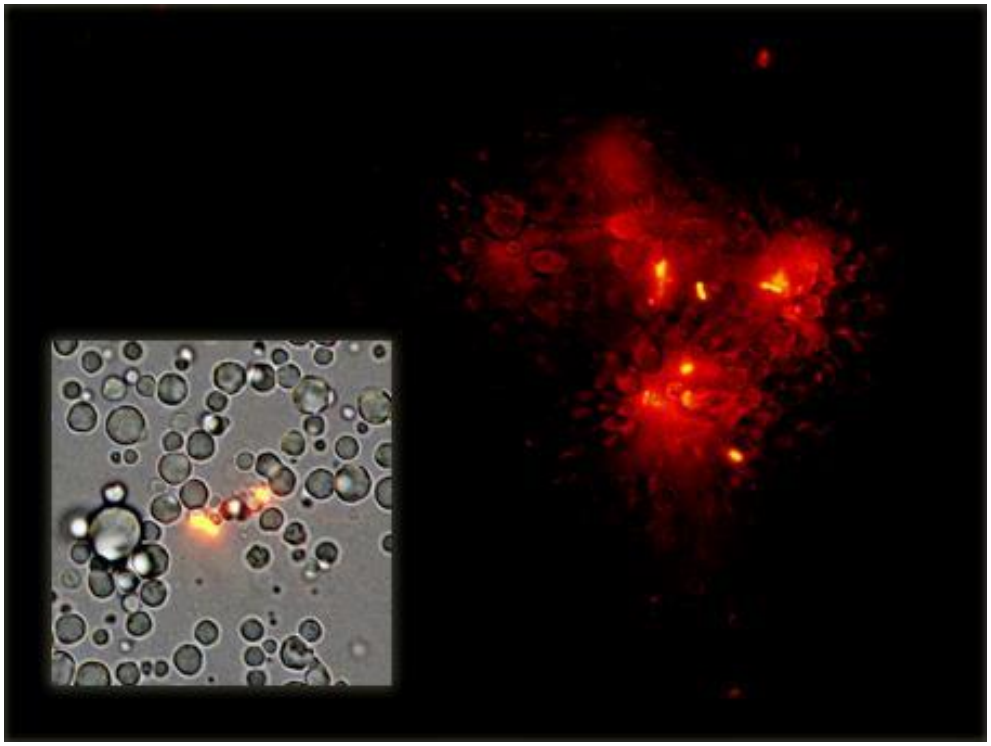

Department of Molecular Sciences
Independent project • 15 hec • First cycle, G2E
Molecular Sciences, 2017:22
Uppsala 2017

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Credits: 15 hec
Level: First cycle, G2E
Course title: Independent Project in Food Science - Bachelor Thesis
Course code: EX0669
Programme/education: Exchange programme

Place of publication: Uppsala
Year of publication: 2017
Cover picture: Captured by W. A. Guwanth Indramal Wanigasuriya, Swedish University of Agricultural Sciences, Department of Molecular Sciences
Title of series: Molecular Sciences
Part number: 2017:22
Online publication: http://stud.epsilon.slu.se

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Abstract

Microbiota composition and content in raw milk is diverse and crucial for the quality of milk as a raw material and subsequent processing. Investigation and identification of microbiota in raw milk is mainly performed based on molecular-based techniques, which are rapid and accurate. At present, defatted milk is used to isolate milk microbiota. A recent study (unpublished) showed that a significant part of milk microbiota is associated with milk fat fraction. Therefore, present study endeavour to continue and confirm the association of microbiota, visually using epifluorescence microscope. A model study was designed to investigate the distribution of recombinant *Lactobacillus reuteri* in bovine milk varied with three levels of fat content (~0.1%, 1.5% and 3.0%). Two fluorescently active recombinant bacterial strains of *L. reuteri* (6475 mCherry and R2LC mCherry) and their wild-type (fluorescently inactive) were cultured in milk. Dilution series for each strains were made by respective cultures and bacteria cells were counted by suitable dilutions to make stock solutions. Milk samples were spiked using stock solutions of bacteria and observed through a fluorescence microscope using UV light. The recombinant bacteria were observed with red light fluorescence filter (Cy3 red filter) and the epifluorescent images captured by a high-definition colour camera (Nikon DS-Fi2) which was attached to the fluorescence microscope. The results from the fluorescent labelled microscopic images showed that *L. reuteri* 6475 mCherry and *L. reuteri* R2LC mCherry strains were associated with milk fat and they were attached to milk fat globules without being free in milk serum. In conclusion, *Lactobacillus reuteri* showed a greater affinity to milk fat fraction in bovine milk than serum fraction. Thus, the results confirmed the previous study and whole milk based microbiota investigation in raw milk should be encouraged as the defatted milk based techniques have the tendency to underestimate the microbiota in raw milk.

Keywords: Milk fat, *Lactobacillus reuteri*, raw milk, microbiota, fluorescence, microorganisms, bovine milk, fat globules

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Abbreviations

FAO	Food and Agriculture Organization of the United Nations
FFA	Free fatty acids
IM	Intermediate milk
LAB	Lactic acid bacteria
MRS	De Man, Rogosa and Sharpe broth
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
SLU	Swedish University of Agricultural Sciences
SM	Skimmed milk
UV	Ultraviolet
WHO	World Health Organization
WM	Whole milk

1 Introduction

Milk and dairy products are consumed by billions of people around the world every day. Therefore, regarding supply to the global demand for milk, to improve food security, assure good quality and to improve further processing, the world needs up-to-date information on raw milk and dairy products. The composition of milk microbiota can be considered as a direct influencing factor for the quality of raw milk and dairy products (Muehlhoff and FAO, 2013). Many methods have been introduced for various milk analyses through the years. Recently, culture-independent investigations through the direct analysis of DNA (or RNA) have been established as they are rapid, reliable, and effective methods for detection and identification of the microorganisms in dairy products (Quigley et al., 2011). However, milk is difficult to analyse using these molecular techniques as it has a complex media that makes it difficult to extract a high quantity and quality amount of DNA. Especially, milk has Polymerase chain reaction (PCR) inhibitors such as fat, protein and calcium which can interrupt amplification of DNA (Wilson, 1997). Usually, the fat fraction is discarded during DNA extraction in these molecular based methods. A recent study showed that a significant part of milk microbiota is located in milk fat fraction (Caroline Blänning, unpublished). Therefore, defatting milk when using molecular methods for detection of microorganisms can cause an underestimation or zero detection of bacteria that are associated with the fat in milk (Poms et al., 2001; Quigley et al., 2012). This can be lead to biased results in most of research in raw milk and dairy products with microbiota. Furthermore, undetected bacteria that surviving the pasteurisation step would affect the final quality of the dairy products (Boor et al., 1998). Since the current molecular methods use defatted milk samples for analysing microbiota in milk, there is a gap which have to be filled by improving methods used for analysing the microbiota in whole milk samples.

1.1 Aim

The research aims were, to investigate the distribution of *Lactobacillus reuteri* in bovine milk samples and to identify the affinity of *L. reuteri* towards milk fat globules as a model study.

2 Literature review

2.1 Bovine milk composition

Raw milk is a highly nutritious food for human consumption, which can be obtained by dairy animals and humans as well (Quigley et al., 2013b). It contains high amounts of nutrients, including fats, proteins, carbohydrates, vitamins, minerals, and essential amino acids with a near neutral pH and a higher water activity (Quigley et al., 2013b). These conditions provide a suitable growth medium for many microorganisms resulting a complex microbial community in raw milk (Frank, 2007; Quigley et al., 2013a). Approximately, bovine milk consists of water (87%), lactose (4.6%), protein (3.4%), fat (4.2%), minerals (0.8%) and vitamins (0.1%). Thus the composition can be varied due to many factors such as breed, seasonal changes, feeding strategies, management of the cow and lactation stage as well (Månsson, 2008). Milk processors produce various kinds of milk products such as liquid milk, fermented milks, cheeses, butter, ghee, milk powder, condensed milk, cream, whey products etc. (FAO, 2017). Raw milk can be defined as the milk which has not been heated over 40 °C or not undergone any treatment that has a similar effect (FAO and WHO, 2009). According to Vaclavik and Christian (2013), whole cow milk is defined as the lacteal secretion, usually free from colostrum, which is collected by the complete milking of a one or more healthy cows. According to the fat content, milk can be classified for example as whole milk, skimmed milk, semi-skimmed milk, low-fat milk and standardized milk (Muehlhoff and FAO, 2013).

Main structural elements of the milk are fat globules, casein micelles, globular proteins and lipoprotein particles. Milk serum consists of above all elements except fat globules (Pieter Walstra et al., 2005).

2.2 Milk fat

When milk is produced in the mammary gland of mammals, lipids as fat droplets are formed by the endoplasmic reticulum located in the epithelial cells that are in the alveoli. Those fat globules are mostly present as an oil-in-water emulsion in bovine milk. They are enveloped by the plasma membrane of cells during the secretion resulting in fat globules with cell residues. Usually fat globules are arranged and organized structures which are composed of three layers. The three layers are monolayer of polar lipids with proteins around the interior lipid core, a protein layer, and the actual external lipid bilayer around the entire fat globule. The external lipid bilayer is made of proteins, glycoproteins, enzymes, non-polar and polar lipids, and phospholipids. An average size of a fat globule is 4 μm which can be varied between 0.2 to 15 μm. However, only a small number of fat globules can be present with sizes exceeding 10 μm. Approximately 30% of the milk fat globule membrane comprised the lipid fraction which consists of lipids such as 25% of phospholipids, 3% of cerebrosides and 2% of cholesterol. Mainly the other fraction of 70% are proteins which many of them exist as enzymes. The whole milk fat consists of mainly triglycerides, which are in approximately 98% of the lipid fraction while 2% contains of other milk lipids such as diacylglycerol, cholesterol, phospholipids and free fatty acids (FFA). Furthermore, in the milk fat there are Ether lipids, hydrocarbons, flavour compounds, fat soluble vitamins and may contain compounds introduced by the feed (Månsson, 2008; Smoczyński et al., 2012).

2.3 Milk microbiota

The milk from a healthy udder is thought to be sterile and thus contaminated by microorganisms from the environment after milking. These microorganisms include both desirable and non-desirable bacteria, Spreer (1998) suggested that microorganisms can be distinguished in milk as technologically useful, technologically harmful and pathogens. Raw milk has a complex microbial community including industrially important microorganisms that having health-promoting features, related to food quality or safety perspectives. Among those microorganisms, the order of lactic acid bacteria (LAB) has an important role in milk which can result in some desirable fermentative reactions. Generally lactic acid bacteria (Order: *Lactobacillales*) are dominant in bovine milk microbiota including genera: *Lactobacillus***,** *Leuconostoc, Enterococcus* and *Streptococcus* (Quigley et al., 2013). However, there are other microorganisms, such as *Pseudomonas* spp., *Acinetobacter* spp., yeasts types and moulds, as well.

2.3.1 Importance of milk microbiota

The milk microbiota composition directly effects the quality of the raw milk and the subsequent development of dairy products. Technologically useful microorganisms can perform processes such as acidification, formation of gas; flavour; texture; taste etc. and protein denaturation (cheese ripening). Technologically harmful microorganisms give negative impacts on quality and properties of milk such as off flavour, unpleasant taste, colour changes and milk precipitation etc. Pathogens can cause foodborne illnesses and toxicities (Quigley et al., 2013; Spreer, 1998). LAB are industrially one of the most important group of microorganisms for milk and dairy products such as cheese, yoghurt, buttermilk, kefir etc. Furthermore, LAB can preserve nutrients in milk and prolong shelf life of the milk. LAB are used extensively in food fermentations and their proteolytic activity is more important in producing flavours for final products (Gemechu, 2015).

2.4 *Lactobacillus reuteri* bacteria

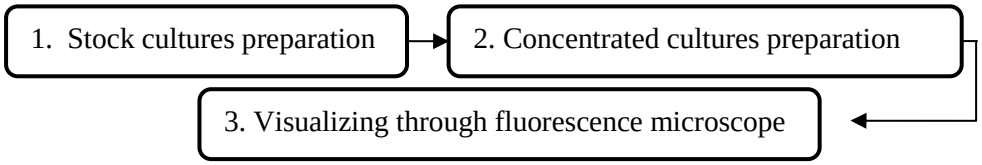
Lactobacillus reuteri is a bacteria species which belongs to the major lactic acid producing genus; *Lactobacillus*. It is a gram positive, rod shaped bacterium (Britton, 2017) which resides naturally in the gastrointestinal tracts of humans and most other animals as an obligately heterofermentative bacteria (Talarico and Dobrogosz, 1989). *L. reuteri* is also found in naturally discharged fluids from utero-vaginal tract and mammary glands as well (Casas and Dobrogosz, 2000). Some strains of *L. reuteri* have been used as probiotics as they are being good health-promoters (Schreiber et al., 2009). *L. reuteri* is one of the most studied LAB species for its capabilities (Karimi et al., 2016). *L. reuteri* 6475 mCherry and *L. reuteri* R2LC mCherry are the recombinant strains that are made by introducing a reporter protein with bacterial plasmids. Antibiotic resistance genes were introduced to those plasmids in order to improve the fitness of their host. Therefore, the recombinant strains are erythromycin dependant (Karimi et al., 2016). Nowadays, there are many different techniques for detection of microbiota in milk. As *L. reuteri* 6475 mCherry and *L. reuteri* R2LC mCherry are able to produce red fluorescence protein, fluorescence microscopy can be used as a detection method.

2.5 Different techniques for detection of microbiota in milk

The composition analyses of milk microbiota can be divided into two categories. They are culture dependent genotyping methods and culture independent molecular methods (Quigley et al., 2011). Culture dependent genotyping methods are “Random Amplified Polymorphic DNA” and “Restriction Fragment Length Polymorphisms” that depend on the isolation, and cultivation of microorganisms before investigate according to their phenotypes or genotypes. However, these methods could have low accuracy and low sensitivity when some microorganisms exists in less numbers (Hugenholtz et al., 1998; Quigley et al., 2011). Therefore, culture independent molecular methods have become more popular. There are many molecular based methods according to Quigley et al. (2011) such as “Denaturing or Temporal Temperature Gradient Gel Electrophoresis”, “Single Stranded Conformation Polymorphisms”, “Real-Time PCR”, “Intergenic Transcribed Spacer Analysis”, “Fluorescence In Situ Hybridization”, “Denaturing High Performance Liquid Chromatography” etc.

3 Materials and Methods

The method development for visualizing fluorescently labelled bacteria was optimized through three major phases as followed.



In the first phase, bacteria culturing, induction with SppIP peptide and, re-culturing of bacteria strains were consisted. The second phase consisted of bacteria cell harvesting, determining cell density and preparation of 10^9 cell concentration cultures of bacterial strains. The third phase consisted of spiking of milk samples using bacteria stock cultures, and observation of the spiked milk through fluorescence microscope.

3.1 Growth medium and solutions

3.1.1 Broth

De Man, Rogosa and Sharpe (MRS) Broth was used to culture each bacterium species. MRS Broth was prepared according to the manufacturer's instructions. Briefly, an amount of 300 ml was prepared and split into forty culture tubes containing 8 ml each (This amount of broth was for the whole experimental period). Those broth containing tubes were autoclaved at 121°C for 30 minutes (CertoCLAV A-4050 autoclave, CertoClav Sterilizer GmbH, Traun, Austria) and stored at 4 °C until further use.

3.1.2 Erythromycin solution

An erythromycin solution was prepared by using 10 ml of 70% ethanol and 100 mg of erythromycin in order to make a 10 mg/ml concentrated solution. The ingredients were mixed in a 15 ml centrifuge tube and vortexed. Thereafter the erythromycin solution was stored at 4 °C for further use.

3.1.3 Phosphate-buffered saline (PBS) solution

A phosphate-buffered saline (PBS) solution (pH 7.2) was prepared according to the standard protocol (Cold spring harber PBS protocol). The solution was autoclaved at 121 °C for 30 minutes and stored at 27 °C.

3.1.4 SppIP inducing peptide solution

An amount of 0.5 mg of dried powder SppIP inducing peptide was dissolved in 1 ml of PBS solution and thereafter diluted with PBS solution in order to get a stock solution with concentration of 50 μg/ml. This solution was stored at -20 °C for further use.

3.2 Bacteria and strains

Four different *L. reuteri* strains were used in the present study, including two recombinant strains (*Lactobacillus reuteri* 6475-mCherry, *Lactobacillus reuteri* R2LC-mCherry) and their wild-type strains (*Lactobacillus reuteri* 6475 WT, *Lactobacillus reuteri* R2LC WT). The two recombinant strains are fluorescently active and therefore, emit fluorescence when exposed to the UV light. The wild-type strains, which are not fluorescently active, were used as negative controls in the experiment.

3.3 Milk samples

The milk used in this study was bulk milk obtained from Lövsta, the Swedish Livestock Research Centre. Skimmed milk was made at our food science dairy lab. The samples were stored in a -20 °C room as aliquots of whole milk and skimmed milk until use. Bovine (Holsteins Swedish Friesian) milk, varied with two levels of fat content (Table 1), was used in the study. The milk component percentages were calculated from a portion of the same bulk milk samples analysed in a previous study in the lab where ten samples for each milk type were used to calculate means.

Table 1. *Types of milk used to make spike-in samples*

Milk type	Fat %	Protein %	Lactose %	Solids %
Whole milk	2.68 ±0.57	3.45 ±0.09	4.77 ±0.04	11.70 ±0.57
Skimmed milk	0.10 ±0.01	3.49 ±0.05	4.90 ±0.01	9.40 ±0.05

Values are mean ±SE

Three combinations of spike-in milk samples were prepared as whole milk (WM) sample skimmed milk (SM) sample and intermediate milk (IM) sample, which contain whole milk and skimmed milk at a ratio of 1:1 (v/v).

3.4 Stock cultures preparation of strains

3.4.1 Inoculation of bacteria and overnight cultures

The four strains were inoculated separately into 8 ml of MRS broth, using inoculation loops. Erythromycin solution was added to the tubes which contained recombinant bacteria at a final concentration of 10 µg/ml. Subsequently, the four culture tubes were kept overnight in 37°C. The overnight cultures were diluted to OD600 of 0.2-0.3 using UV/visible spectrophotometer (Ultrospec™ 1100 pro UV/visible spectrophotometer, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Optical densities were adjusted for obtaining the appropriate concentrations. Thereafter, 8 µl from 50 µg/ml concentrated SppIP inducing peptide was added into each mCherry cultures to a final concentration of 50 ng/ml of SppIP inducing peptide. Then, the cultures were incubated at 37°C for 20 hours.

3.4.2 Bacteria cell harvesting, determining cell density and preparation of stock cultures

At the end of 20 hours of incubation, each culture solutions were divided into six 2 ml Eppendorf tubes separately. The samples were centrifuged for 20 minutes, 5000 × *g* at 4°C (Himac CT15RE, Hitachi Koki Co., Ltd., Japan). Afterwards the supernatants were removed carefully without disturbing the pellets and the pellets were re-suspended using 1 ml PBS, pH 7.2 and vortexed. Consequently, six concentrated bacteria solutions were obtained. A dilution series with concentration 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} was made in order to identify the suitable dilution for counting bacteria cells. Numbers of bacteria cells were counted using a Petroff-Hausser counting chamber (Neubauer improved cell counting Helber chamber, C.A. Hausser & Son., Philadelphia, USA) via an Olympus CHS microscope (Olympus optical co., ltd., Japan)

equipped with an A40PL 40× objective and CWHK 10× eyepiece. After cell counting, cell concentration was calculated according to the Equation given below.

$$\text{Cells / ml} = \frac{\text{Number of cells counted}/16}{0.00005} \times 1000 \times \text{dilution factor}$$

Where, number of cells counted = number of cells counted in 16 small squares; 0.00005 = volume of the smallest squares on the chamber; 1000 = to convert the amount into ml, Dilution factor = Solution volume / aliquot volume (Appendix 4)
Diluted sample 10^{-2} concentration was chosen for cell counting and the concentration of 10^9 cell/ml stock culture solutions were made from those samples which were used to count bacterial cells.

3.5 Spiking milk samples with bacteria stock cultures

Three samples from each strain was made by pipetting 300 µl of stock culture solutions to 1.5 ml Eppendorf tubes containing 700 µl of milk. Each strain was spiked in to 700 µl of WM, SM and IM (Table 2).

Strain	Spiked sample number and type of milk		
L. reuteri 6475 mCherry	S_1 -WM	S_5 - SM	S_9 - IM
L. reuteri R2LC mCherry	S_2 - WM	S_6 - SM	S_{10} - IM
L. reuteri 6475 wild type	S_3 - WM	S_7 - SM	S_{11} - IM
L .reuteri R2LC wild type	S_4 - WM	S_8 -SM	S_{12} - IM

WM- Whole milk, SM- Skimmed milk, IM- Milk mixture (1:1)
Table 2. *Sample design of spiked milk with bacterial strains*

3.6 Observation of samples through fluorescence microscope

The spiked milk samples were shaken at 4 °C for 1 hour. Thereafter, samples were studied using a fluorescence microscope (Nikon Eclipse-Ni Light Microscope with connected Nikon Intensilight C-HGFI mercury lamp). The epifluorescence images and optical microscopic images were observed at 40× and 60× (oil immersed lens) magnifications and images captured by a high-definition colour camera (Nikon DS-Fi2, Nikon corporation, Japan) which was attached to the fluorescence microscope. The mCherry fluorescence bacteria were observed with red light fluorescence filter (Cy3 red filter, excitation 512-552 nm, emission 565-615 nm). The images were processed by Nikon NIS-Elements imaging software and rearranged by ImageJ image processing software (ImageJ 1.51q).

3.6.1 Image overlaying to obtain fluorescent labelled images

Image overlaying was more important in order to get fluorescent labelled images of bacterial strains. As shown in the Figure 1, initially bacteria were located by observing a sample through red light fluorescence filter with fluorescence microscope. Then, an image was taken. Afterwards, switching to normal optical light microscope view (without red filter) another image was taken without changing the previous position. The two images were merged to obtain a fluorescent labelled image using the aforesaid software.

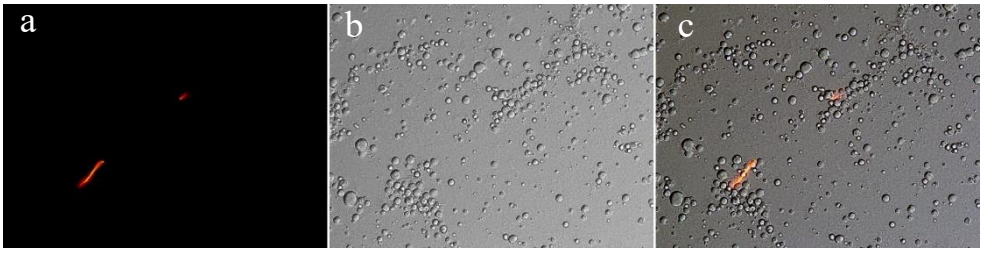


Figure 1. Steps in image overlaying for a milk sample a) Epifluorescence image b) Optical microscopic image c) Merged image of a and b: Fluorescence labelled image (60×). Reddish colour spots are fluorescent active bacterial cells.

4 Results

4.1 Culturing of bacterial strains

The four cultured *Lactobacillus reuteri* strains showed fine growth in MRS broth tubes which resulted in high optical density values and cell counts.

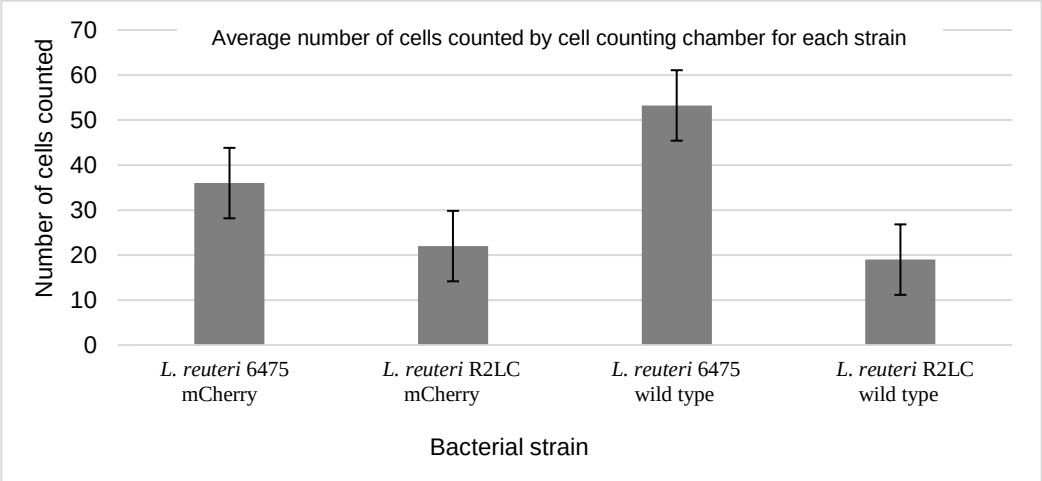


Figure 2. Average number of cells counted by cell counting chamber for each strain. Four samples for each strain were used for calculations.
(The standard errors for each strain are shown in black bars)

The growth can be interpreted by the cell count of the bacterial strains which showed significant differences among the strains except for between *L. reuteri* R2LC strains. The highest growth was observed for *L. reuteri* 6475 wild type strain ($P<0.05$) whereas the second higher growth was shown by *L. reuteri* 6475 mCherry (Figure 2). Moreover, the results showed that (Figure 2) *L. reuteri* 6475 strains had a higher growth ($P<0.05$) than *L. reuteri* R2LC strains. However, the growth of the two recombinant bacterial strains *L. reuteri* 6475 mCherry and *L. reuteri* R2LC mCherry showed a slight reduction compared to the related wild type strains. Relevant data and statistical analyses are shown in Appendix 1.

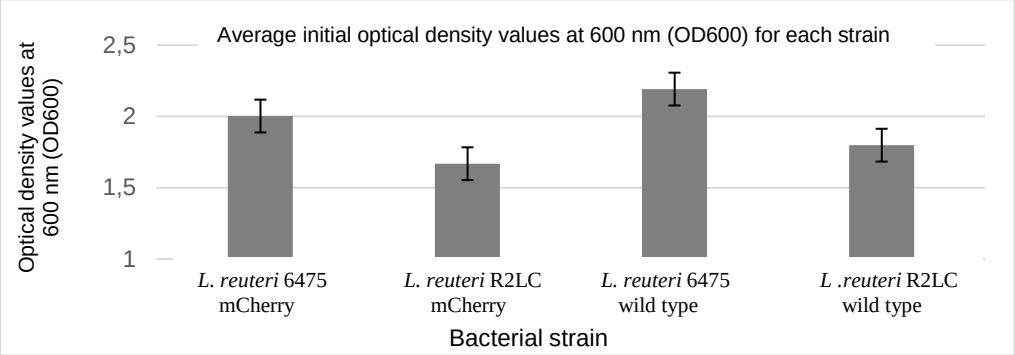


Figure 3. Average optical density values at 600 nm (OD600) for each bacterial strain in initial measurements. Four samples for each strain were used for calculations.
(The standard errors for each strain are shown in black bars)

Comparatively, the mean optical density values (OD600) for each bacterial strain were not significantly different. Thus there can be seen a trend in mean values which resembles the results in the figure 2.

4.2 Epifluorescence microscopic images of *L. reuteri* strains

4.2.1 *L. reuteri* wild-type strain images

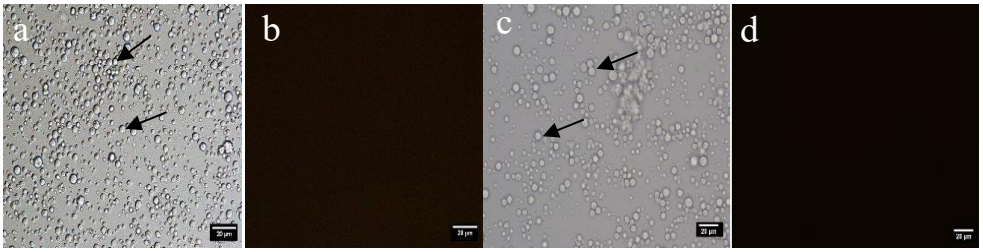


Figure 4. Images of *L. reuteri* 6475 wild type strain of WM sample (60×). Image a) was captured as an optical microscopic image which can be seen with fat globules as circular droplets; b) Epifluorescence image of the same sample where cannot see any natural background fluorescence. Images of *L. reuteri* R2LC wild type strain of a WM sample (60×). Image c) was captured as an optical microscopic image which can be seen with fat globules as circular droplets; d) Epifluorescence image of the same sample where cannot see any natural background fluorescence. Due to absence of fluorescence b) and d) images shows black colour background.

The results obtained from *L. reuteri* wild type strains are shown in Figure 4. The epifluorescent images with black backgrounds show that there was not any natural background fluorescence given by wild type strains. Black arrows show fat globules which can be seen as circular droplets.

4.2.2 *L. reuteri* 6475 mCherry images

L. reuteri 6475 mCherry strain spiked milk samples were studied and results are shown in Figure 5 and in Appendix 2 (Figure 1.1, Figure 1.2, Figure 1.3).

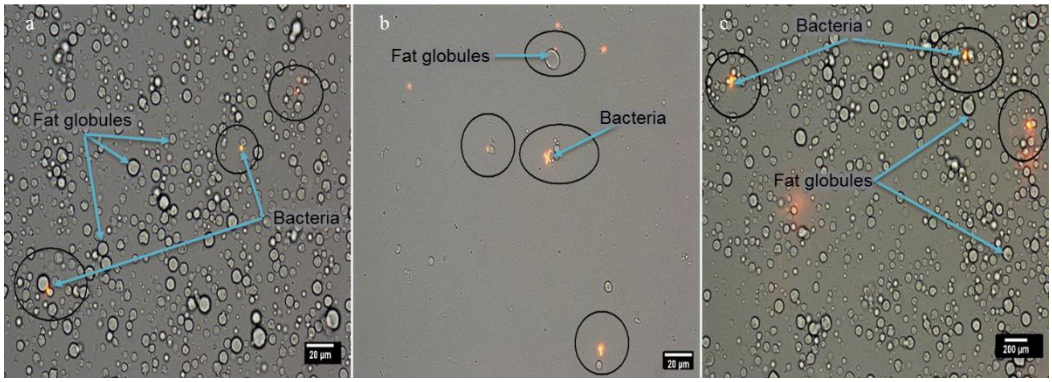


Figure 5. Fluorescence labelled images of *L. reuteri* 6475 mCherry strain (60×) a) is an image of the strain in WM sample; bacteria attached to fat globules are shown in circles and some fat globules are shown by arrows. b) is an image of the strain in SM sample; bacteria attached to fat globules are shown in circles and some fat globules are shown by arrows. c) is an image of the strain in IM sample; bacteria attached to fat globules are shown in circles and some fat globules are shown by arrows.

L. reuteri 6475 mCherry bacteria which attracted to the fat globules can be seen in the circled areas of Figure 5. The bright reddish colour spots are the florescent *L. reuteri* 6475 mCherry cells. The most of bacteria can be seen attached to fat globules.

4.2.3 *L. reuteri* R2LC mCherry images

The results obtained from *L. reuteri* R2LC mCherry strain of different milk samples are shown in Figure 6 and Appendix 3 (Figure 2.1, Figure 2.2, Figure 2.3). *L. reuteri* R2LC mCherry strain bacteria were mostly appeared as individuals or densely clotted forms.

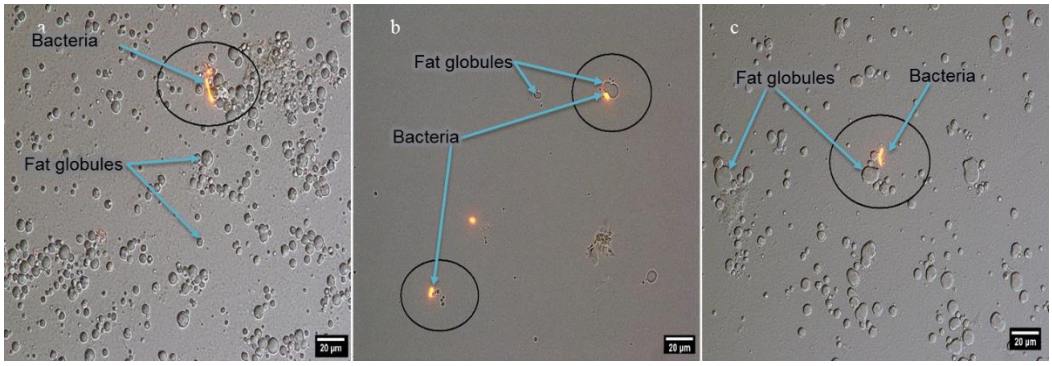


Figure 6. Fluorescence labelled images of *L. reuteri* R2LC mCherry strain (60×). a) is an image of the strain in WM sample; bacteria attached to fat globules are shown in circles and some fat globules are shown by arrows. b) is an image of the strain in SM sample; bacteria attached to fat globules are shown in circles and some fat globules are shown by arrows. c) is an image of the strain in IM sample; a bacterium attached to fat globules is shown in a circle and some fat globules are shown by arrows.

L. reuteri R2LC mCherry strain also existed together with fat globules which can be seen in the circled areas of Figure 6. Similarly, the reddish colour spots are the bacteria which attracted to the fat globules.

5 Discussion

The aim of this project was to investigate the distribution of *Lactobacillus reuteri* in bovine milk fraction and also to identify the affinity of *L. reuteri* to the milk components in the raw milk. Since some studies have shown that a significant part of milk microbiota is located in milk fat fraction, this was done in order investigate how *L. reuteri* is located in milk fractions.

The initial task of the project was to culture four *L. reuteri* strains for spiking milk samples. According to the numbers of cell counted for each strain, higher growths of *L. reuteri* 6475 strains compared ($P<0.05$) with *L. reuteri* R2LC strains and slightly impaired growths of the two recombinant strains compared ($P<0.05$) with wild type strains, were resembled the results in previous findings of Karimi et al. (2016). The slightly impaired growth of recombinant strains may be due to their cellular modifications such as introduction of recombinant vector. However, the results of bacterial cell counts were different from their growths showed in the initial absorbance values. *L. reuteri* R2LC strains showed lesser number of counted bacteria than *L. reuteri* 6475 strains. A possible reason for that could be the clotting behaviour of *L. reuteri* R2LC strains which makes unequal distribution on the cell counting chamber. Also, the bacterial clots made it difficult to count bacteria as they could not be distinguished as separate cells. The cell counting chamber is not a fully accurate method in order to get exact concentrations. Additionally, dead cells, and live cells were difficult to distinguish by the cell counting chamber. Therefore, staining bacteria could be a good way to make counting easy on the cell counting chamber.

Making the dilution series only 10^{-1}, 10^{-2}, 10^{-3} concentrations are enough for cell counting. Frequently, 10^{-2} concentration was the perfect sample to count bacteria. Reculturing bacteria for repeating the experiment was done using old overnight cultures. It resulted in high amount of non-fluorescent bacteria to show up in milk samples (spiked with recombinant bacteria) when observing through the microscope. This could be happening when recombinant plasmids do not persist in bacteria due to the unavailability of sufficient amount of antibiotics. Another reason for that could be contamination of bacteria from environment with availability of fine growth media:

MRS broth. Therefore, using fresh, froze stock solutions to make overnight cultures could be a better option in order to minimize contaminations from environment. It is obvious that erythromycin should be added always to the recombinant strains when having an incubation to assure plasmid persistency. In some occasions, focusing on both fat globule and attached bacteria was difficult as the bacteria was on the fat globule or beneath the fat globule which makes them into two level for focusing. Moreover, some small fat globules and attached bacteria were difficult to distinguish separately. Therefore, examine the fat stained samples with fluorescent bacteria through confocal microscope would be a better option.

In addition to the above future perspectives on improvements, many implications could be done as suggested hereafter. This method can be easily used for quantification of bacteria using fluorescent images with scale and imageJ software. Furthermore, this can be used as an efficient method for visualization of milk microbiota in raw milk. The affinity of bacteria may due to their hydrophobic qualities or may be that fat globule membrane act as a substrate for the bacteria. Therefore, expanding this research to analyse attraction towards casein micelles, water (hydrophilic and hydrophobic qualities), and other components could provide more interesting research findings. In addition to that, it is possible to investigate specific binding sites of bacteria with fat globule membrane using scanning and transmission electron microscopes.

5.1 Conclusion

This research investigated the distribution of *Lactobacillus reuteri* in bovine milk samples and whether there were any affinity of *L. reuteri* towards milk fat globules. The fluorescent labelled images were shown that most of the bacteria were together with fat globules and not freely floating in milk serum. Moreover, the bacteria were dispersed in the locations where milk fat globules existed. In conclusion, it means that *L. reuteri* 6475 mCherry and *L. reuteri* R2LC mCherry were associated with milk fat fraction. Furthermore, the images were shown that the bacteria adhered to milk fat globules which can be concluded that there is an affinity of *L. reuteri* 6475 mCherry and *L. reuteri* R2LC mCherry towards milk fat in the milk.

Acknowledgements

This bachelor thesis is submitted to the Department of Molecular Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden for the accomplishment of International Credit Mobility (ICM) programme. This program is connected to Erasmus+ action within SLU and University of Peradeniya, Sri Lanka. I would sincerely express my gratitude and I am grateful to the following supervisors for their enormous support and collaboration given to make this project a success. Associate Professor Monika Johansson, the main supervisor at SLU is specially thanked for the academic support given to complete the research project. Your view points, supervision and expertise made this project successful. Senior Lecturer, Dr. Janak K. Vidanarachchi is specially acknowledged for the supports since from the beginning of the mobility programme. I am very glad to be supervised and receive guidance from you throughout the project. I really appreciate the support and advices from Mr. Hasitha Priyashantha, the co-supervisor of my research and is also specially thanked. Also I would like to thank co-supervisor Dr Li Sun for the advices and support during the research. The reporter construct of *L. reuteri* 6475-mCherry, *L. reuteri* R2LC-mCherry, *L. reuteri* 6475 WT, *L. reuteri* R2LC WT and SppIP induction peptide were kindly provided by Dr. Stefan Roos and Prof. Hans Jonsson and I am thankful to them also. Professor Åse Lundh is specially thanked for giving this great opportunity to engage in this research. Also I would like to express my sincere gratitude to Dr. Daniel Johansson and PhD student Anton Pallin for the technical support and guidance given for fluorescence microscope and for cell counting chamber respectively. Also, I would like to thank to the mobility team at SLU for arranging the wonderful study aboard period in Sweden. Also I am grateful to Prof. Lena Dimberg for evaluating this research. I am thankful for Mrs. Shishanthi Jayarathna, Ms. Yashaswini Nagaraj and Ms. Thilini Nuwandhara for the meaningful discussions and support given during the research period. I highly appreciate the contribution of everyone, who supported directly and indirectly to make this thesis a success. Last but not least, I would like to thank to family and intimate friends for being supportive and encouraging to complete the project. Hope that these findings will be useful for further researches. It has been a pleasure to be a part of your group.

References

Boor, K.J., Brown, D.P., Murphy, S.C., Kozlowski, S.M., Bandler, D.K., 1998. Microbiological and chemical quality of raw milk in New York State. *J. DAIRY SCI.* 81, 1743–1748.

Britton, R.A., 2017. Chapter 8 - *Lactobacillus reuteri*, in: Floch, M.H., Ringel, Y., Walker, W.A. (Eds.), The Microbiota in Gastrointestinal Pathophysiology. Academic Press, Boston, pp. 89–97.

Caroline Blänning, 2017. DNA based analyses of microbiota in fat fraction of raw milk. Unpubl. Data, Master thesis, SLU.

Casas, I.A., Dobrogosz, W.J., 2000. Validation of the probiotic concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals. *MICROB. ECOL. HEALTH DIS.* 12, 247–285.

FAO, F., 2017. Dairy production and products: Types and characteristics [WWW Document]. URL http://www.fao.org/agriculture/dairy-gateway/milk-and-milk-products/types-and-characteristics/en/#.WedCg2iCzIU (accessed 10.18.17).

Gemechu, T., 2015. Review on lactic acid bacteria function in milk fermentation and preservation. *AFR. J. FOOD SCI.* 9, 170–175.

Hugenholtz, P., Goebel, B.M., Pace, N.R., 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. BACTERIOL.* 180, 4765–4774.

Karimi, S., Ahl, D., Vågesjö, E., Holm, L., Phillipson, M., Jonsson, H., Roos, S., 2016. *In vivo* and *in vitro* detection of luminescent and fluorescent *lactobacillus reuteri* and application of red fluorescent mcherry for assessing plasmid persistence. *PLOS ONE 11*, e0151969.

Månsson, H.L., 2008. Fatty acids in bovine milk fat. *FOOD NUTR. RES.* 52, 1821.

Muehlhoff, E., FAO (Eds.), 2013. Milk and Dairy Products in Human Nutrition. FAO, Rome.

Pieter Walstra, Jan T. M. Wouters, Tom J. Geurts, 2005. Dairy Science and Technology, Second Edition [WWW Document]. CRC Press. URL

https://www.crcpress.com/Dairy-Science-and-Technology-Second-Edition/Walstra-Walstra-Wouters-Geurts/p/book/9780824727635 (accessed 10.18.17).

Poms, R., Glössl, J., Foissy, H., 2001. Increased sensitivity for detection of specific target DNA in milk by concentration in milk fat. *EUR. FOOD RES. TECHNOL.* 213, 361–365.

Quigley, L., O'Sullivan, O., Beresford, T. p., Paul Ross, R., Fitzgerald, G. f., Cotter, P. d., 2012. A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese. *J. APPL. MICROBIOL.* 113, 96–105.

Quigley, L., O'Sullivan, O., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D., 2011. Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *INT. J. FOOD MICROBIOL.* 150, 81–94.

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D., 2013. The complex microbiota of raw milk. *FEMS MICROBIOL. REV.* 37, 664–698.

Schreiber, O., Petersson, J., Phillipson, M., Perry, M., Roos, S., Holm, L., 2009. *Lactobacillus reuteri* prevents colitis by reducing P-selectin-associated leukocyte- and platelet-endothelial cell interactions. *AM. J. PHYSIOL. - GASTROINTEST. LIVER PHYSIOL.* 296, G534–G542.

Smoczyński, M., Staniewski, B., Kiełczewska, K., 2012. Composition and Structure of the Bovine Milk Fat Globule Membrane—Some Nutritional and Technological Implications. *FOOD REV.* Int. 28, 188–202.

Spreer, E., 1998. Milk and Dairy Product Technology. CRC Press.

Talarico, T.L., Dobrogosz, W.J., 1989. Chemical characterization of an antimicrobial substance produced by *Lactobacillus reuteri*. *ANTIMICROB. AGENTS CHEMOTHER.* 33, 674–679.

Vaclavik, V.A., Christian, E.W., 2013. Essentials of Food Science. Springer Science & Business Media.

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. *APPL. ENVIRON. MICROBIOL.* 63, 3741–3751.

FAO & WHO**.** 2009. Code of hygienic practice for milk and milk products. *CODEX ALIMENTARIUS.* CAC/RCP 57-2004. Available at: http://www.codexalimentarius.org/download/standards/10087/CXP_057e.pdf. (accessed 18.09.2012).

Appendix 1 Data and statistical analyses of graphs

Optical density values at 600 nm (OD600) for each strain

Table 1.1. *Average optical density values at 600 nm (OD600) for each bacterial strain in initial measurements*

Bacterial strains	**Optical density values at 600 nm (OD_{600})**			
***L. reuteri* 6475 mCherry**	2.210	1.534	2.142	2.125
***L. reuteri* R2LC mCherry**	1.215	1.414	2.374	1.671
***L. reuteri* 6475 wild type**	2.454	1.654	2.136	2.523
***L .reuteri* R2LC wild type**	1.535	1.556	2.211	1.892

One-way ANOVA: optical density values at 600 nm (OD600) for each strain

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	4	L. reuteri 6475 mCherry, L. reuteri R2LC mCherry, L. reuteri 6475 wild type, L .reuteri R2LC wild type

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	0.6345	0.2115	1.38	0.297
Error	12	1.8431	0.1536		
Total	15	2.4776			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.391908	25.61%	7.01%	0.00%

Means

Factor	N	Mean	StDev	95% CI
L. reuteri 6475 mCherry	4	2.003	0.315	(1.576, 2.430)
L. reuteri R2LC mCherry	4	1.669	0.506	(1.242, 2.095)
L. reuteri 6475 wild type	4	2.192	0.396	(1.765, 2.619)
L .reuteri R2LC wild type	4	1.798	0.320	(1.372, 2.225)

Pooled StDev = 0.391908

Number of cells counted by cell counting chamber for each strain

Table 1.2. *Cell count and concentrations for 10-2 concentration of each strain*

Bacterial strain	**Number of cells counted***			
***L. reuteri* 6475 mCherry**	32	45	48	19
***L. reuteri* R2LC mCherry**	31	23	20	14
***L. reuteri* 6475 wild type**	58	47	51	57
***L .reuteri* R2LC wild type**	16	18	25	17

**1 mm × 1 mm area of center square in the counting chamber*

*Statistical analysis for the Table 1.3 is in the next page

One-way ANOVA: number of cells counted by cell counting chamber for each strain

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	4	L. reuteri 6475 mCherry, L. reuteri 6475 wild type, L .reuteri R2LC wild type, L. reuteri R2LC mCherry

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	3676.5	1225.49	24.18	0.000
Error	16	810.8	50.67		
Total	19	4487.2			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
7.11842	81.93%	78.54%	71.77%

Means

Factor	N	Mean	StDev	95% CI
L. reuteri 6475 mCherry	5	36.00	11.51	(29.25, 42.75)
L. reuteri 6475 wild type	5	53.25	4.49	(46.50, 60.00)
L .reuteri R2LC wild type	5	19.00	3.54	(12.25, 25.75)
L. reuteri R2LC mCherry	5	22.00	6.12	(15.25, 28.75)

Pooled StDev = 7.11842

Tukey Pairwise Comparisons
Grouping Information Using the Tukey Method and 95% Confidence

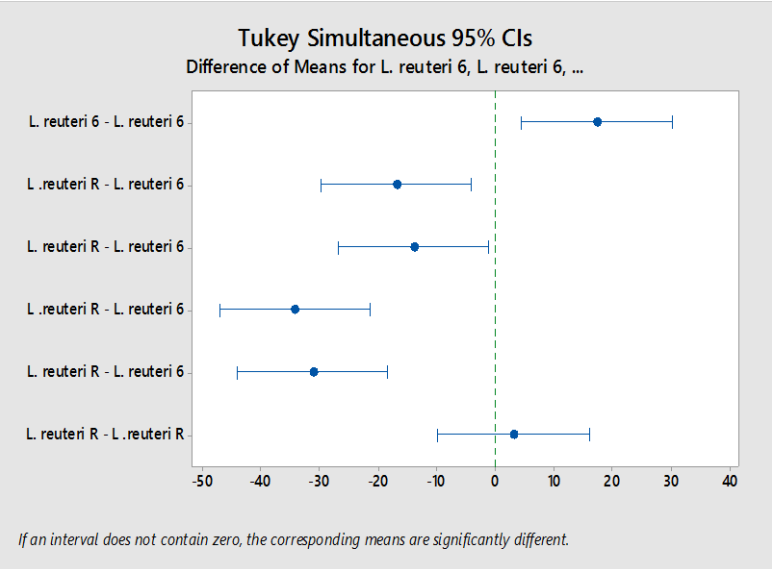
Factor	N	Mean	Grouping		
L. reuteri 6475 wild type	5	53.25	A		
L. reuteri 6475 mCherry	5	36.00		B	
L. reuteri R2LC mCherry	5	22.00			C
L .reuteri R2LC wild type	5	19.00			C

Means that do not share a letter are significantly different.

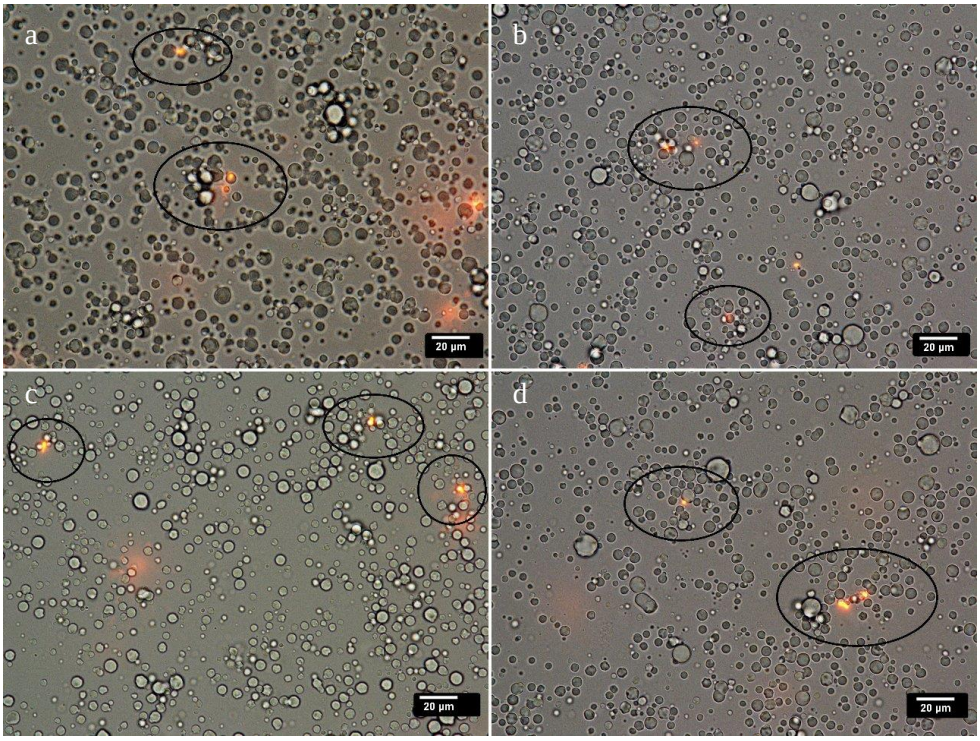
Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
L. reuteri 6 - L. reuteri 6	17.25	4.50	(4.36, 30.14)	3.83	0.007
L .reuteri R - L. reuteri 6	-17.00	4.50	(-29.89, -4.11)	-3.78	0.008
L. reuteri R - L. reuteri 6	-14.00	4.50	(-26.89, -1.11)	-3.11	0.031
L .reuteri R - L. reuteri 6	-34.25	4.50	(-47.14, -21.36)	-7.61	0.000
L. reuteri R - L. reuteri 6	-31.25	4.50	(-44.14, -18.36)	-6.94	0.000
L. reuteri R - L .reuteri R	3.00	4.50	(-9.89, 15.89)	0.67	0.908

Individual confidence level = 98.87%



Appendix 2 *L. reuteri* 6475 mCherry images



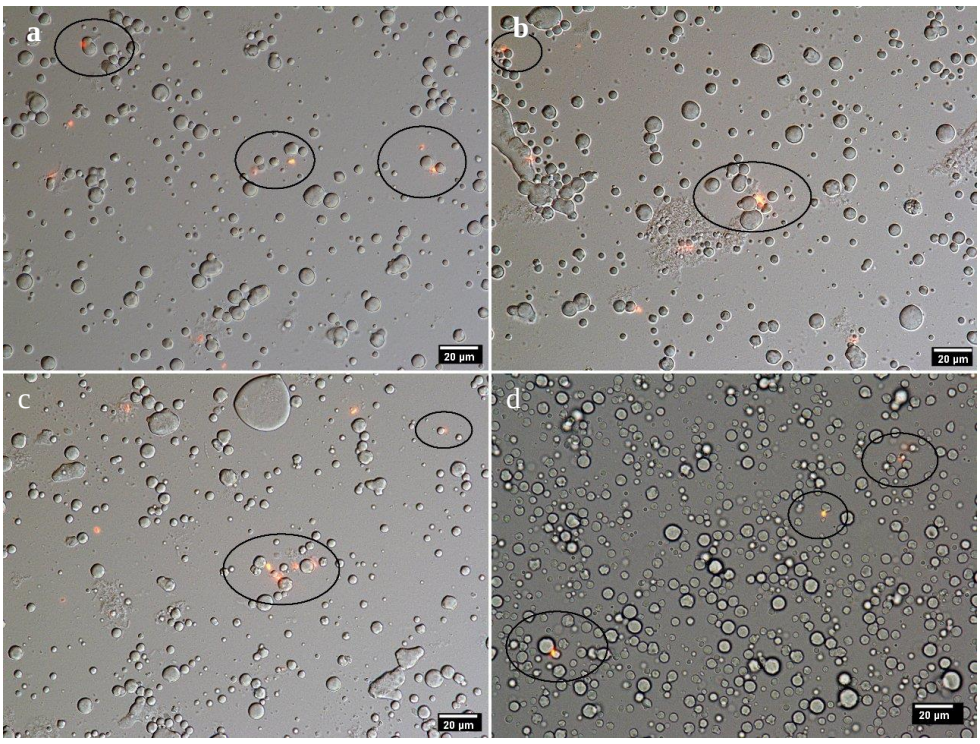


Figure 1.1. Fluorescence labelled images of *L. reuteri* 6475 mCherry strain of a WM sample (60×).

Figure 1.2. Fluorescence labelled images of *L. reuteri* 6475 mCherry strain of an IM sample (60×).

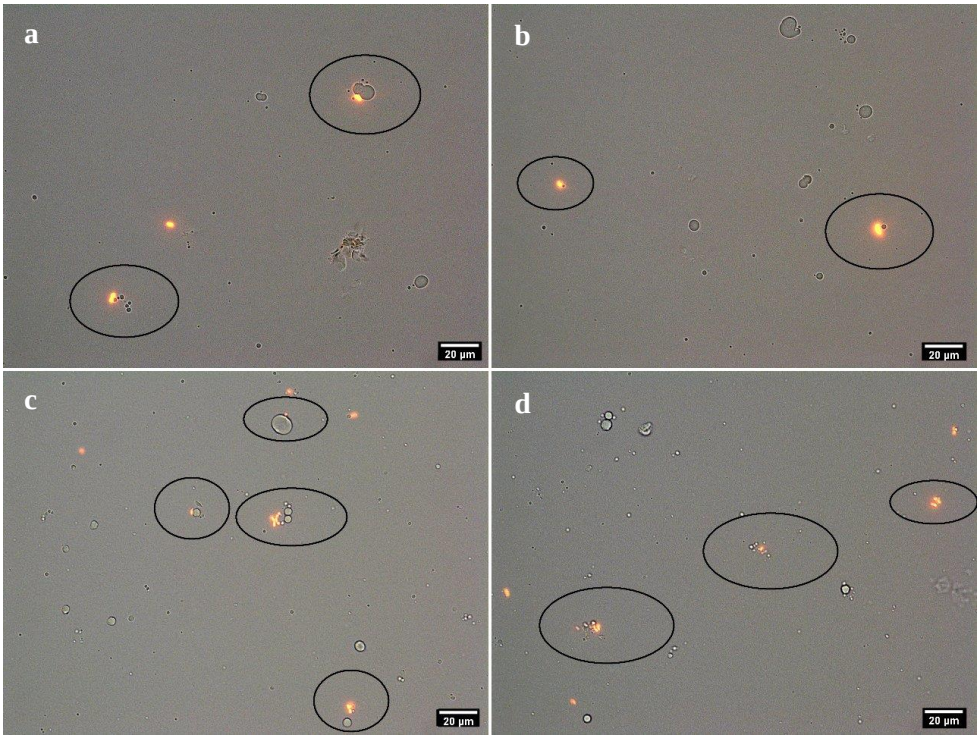


Figure 1.3. Fluorescence labelled images of *L. reuteri* 6475 mCherry strain of a SM sample (60×).

Appendix 3 *L. reuteri* R2LC mCherry images

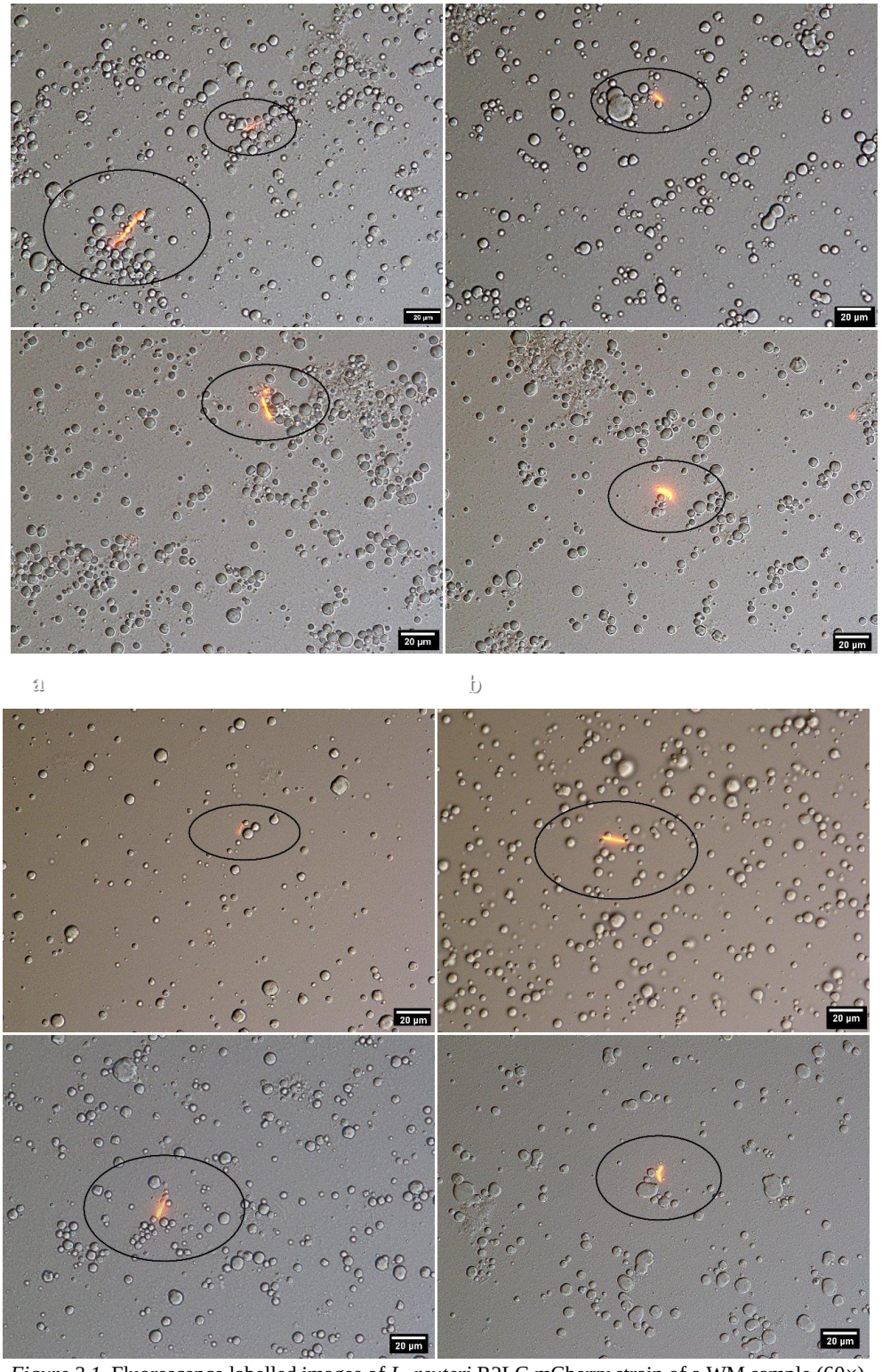


Figure 2.1. Fluorescence labelled images of *L. reuteri* R2LC mCherry strain of a WM sample (60×).

Figure 2.2. Fluorescence labelled images of *L. reuteri* R2LC mCherry strain of an IM sample (60×).

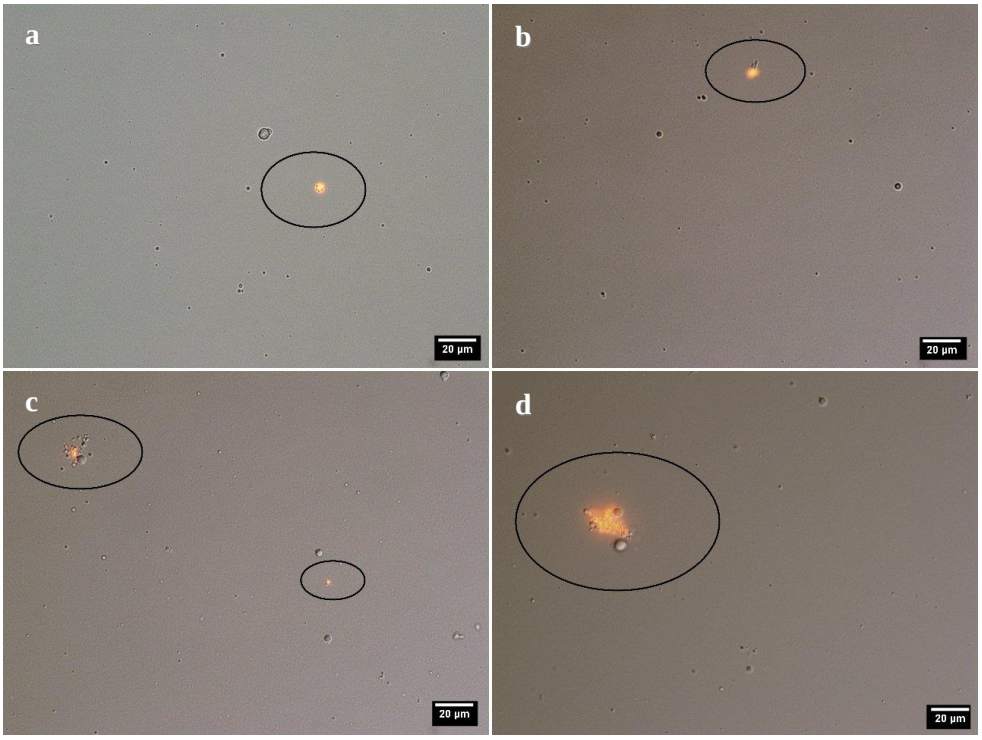


Figure 2.3. Fluorescence labelled images of *L. reuteri* R2LC mCherry strain of a SM sample (60×).

Appendix 4 Neubauer improved cell counting Helber chamber

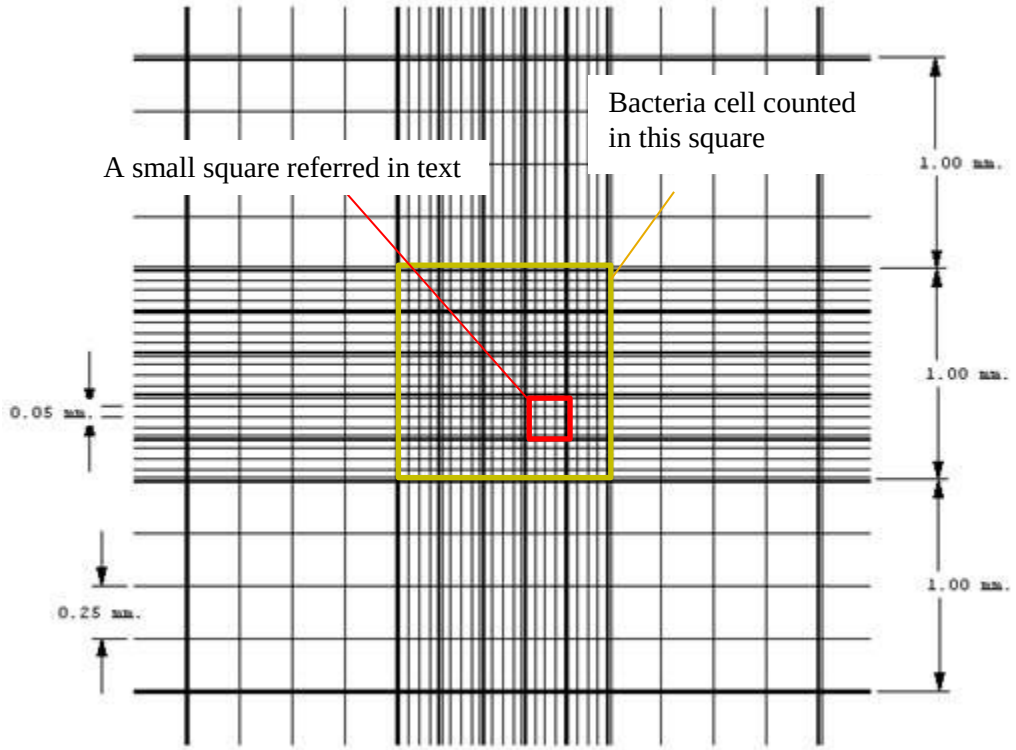


Figure 3. Neubauer Ruling of a Petroff-Hausser counting chamber (Neubauer improved).